Collagen in the scarless fetal skin wound: Detection with Picrosirius-polarization

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Our group has developed an ovine model of deep dermal, partial-thickness burn where the fetus heals scarlessly and the lamb heals with scar. The comparison of collagen structure between these two different mechanisms of healing may elucidate the process of scarless wound healing. Picrosirius staining followed by polarized light microscopy was used to visualize collagen fibers, with digital capture and analysis. Collagen deposition increased with fetal age and the fibers became thicker, changing from green (type III collagen) to yellow/red (type I collagen). The ratio of type III collagen to type I was high in the fetus (166), whereas the lamb had a much lower ratio (0.2). After burn, the ratios of type III to type I collagen did not differ from those in control skin for either fetus or lamb. The fetal tissue maintained normal tissue architecture after burn while the lamb tissue showed irregular collagen organization. In conclusion, the type or amount of collagen does not alter significantly after injury. Tissue architecture differed between fetal and lamb tissue, suggesting that scar development is related to collagen cross-linking or arrangement. This study indicates that healing in the scarless fetal wound is representative of the normal fetal growth pattern, rather than a ''response'' to burn injury. **(WOUND REP REG 2005;13:198–204)**

Collagen is the major component of extracellular matrix and provides tensile strength to skin. During the wound healing process, it acts to modulate cell proliferation and migration and is important in the wound contraction process. The patterns of collagen deposition in healing fetal and adult wounds differ markedly. Fetal skin regenerates collagen fibers in neat, well-organized patterns with close to perfect tissue architecture, whereas postnatal and adult skin heals with collagen laid down in thick disorganized patterns and scar formation.[1] The scarless healing properties of fetal skin are lost in many animal models in late gestation.[2–4] The study of collagen structure, distribution, and levels before and after this critical fetal time point may contribute to our understanding of the process of scarless fetal wound healing.

Of the many different types of collagen identified, fetal skin is known to contain a greater proportion of type III collagen,[5,6] whereas adult skin consists predominantly of type I collagen.[7] After wounding, collagen is deposited in both adult and fetal wounds, but reports of the extent and the types of collagen deposited vary.[6,8–10] This variability may be due to the many different animal models used to study wound healing (rats, rabbits, sheep, pigs, humans) and the various ways in which collagen can be measured.

There are many different assays that detect and quantitate the various types of collagen during wound healing. These include hydroxyproline assays, trichrome dye staining, sponge/tube implantation followed by protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein extraction from whole skin samples followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis,

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Manuscript received: May 3, 2004
Accepted in final form: September 24, 2004
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ISSN: 1067-1927.

immunohistochemistry, and in situ hybridization analysis of mRNA in tissues. Most of these methods are not ideal. Assays and gel analysis involve collection and analysis of skin samples that may include normal control tissue outside the border of the wound. Implanted sponges and tubes are an artificial environment that alter the structure of the skin and may not be truly indicative of the skin's natural ability to deposit collagen in a wound. Immunohistochemistry is currently the most frequently used technique, but this method is dependent on the availability of specific antibodies that will bind to tissue sections from the animal model being used. In this study, the lack of available antibodies for an ovine model of scarless healing necessitated the use of another method to explore the distribution and relative abundance of collagen.

The use of Sirius red dye or Picrosirius to differentiate between the different collagen types in tissue was first described by Junqueira et al.[11] The method is simple, reliable, and inexpensive, while producing images of beautiful colors under polarized light. Collagen has a natural birefringence due to the arrangement of its fibers and this property is enhanced by Sirius Red dye.[12] Under polarized light, the fibers seemingly glow with bright colors against a black background. Type I collagen, which tends to form thick collagen fibers composed of closely packed thick fibrils, birefringes as an intense yellow to red color. Type III collagen forms thin fibers, composed of loosely disposed thin fibrils and has weak green birefringence.[12] Thus the color displayed is a result of the thickness of the fiber, as well as the arrangement of the collagen molecules.[13]

In a previous study, we developed an ovine model of deep dermal burn injury.[14] In this model, a reproducible deep dermal burn was created in the ovine fetus and lamb by application of timed, hot water. This tissue has been examined previously in three different ways: macroscopically, immunohistochemically (staining for α-smooth muscle actin and transforming growth factor-β) and histopathologically, by using a five-point histopathology scoring system for alteration in tissue morphology.[14] Differences were detected between control and scalded skin at all stages in lamb postburn but no difference was detected in the fetal model after day 7, indicating that the fetus heals a deep burn injury in a scarless fashion. The comparison of collagen between the fetal skin, which was found to heal the burn wound without scar, to a postnatal lamb wound that healed the same injury with scar, may give some indication of the role of collagen in the scarless healing of fetal wounds. In this present study, fetal and lamb skin from this previously developed ovine deep dermal burn model were stained with Picrosirius dye and observed under polarized light to detect the structure, distribution, and type of collagen present.

MATERIALS AND METHODS

The skin sections used for this work were obtained from fetal and lamb sheep skin when a scarless burn wound model was created.[14] The University of Queensland Animal Ethics Committee approved all animal wounding protocols and all animals received humane care.

Briefly, pregnant ewes were operated on under sterile conditions to partially deliver the fetus at day 80 gestation. The fetal flank was then burned using hot water (66 °C for 7 seconds) contained in a propylene tube of 1.5 cm diameter, to create a deep dermal partial-thickness burn. The fetus was then returned to the uterus and all surgical incisions closed. A total of 15 fetuses underwent euthanasia at 1, 7, 14, 21, and 60 days after the scalding, with three fetuses terminated at each time point. The lambs were scalded in a similar manner at day 30 after birth, also using hot water (82 °C for 10 seconds) on freshly shaved skin. A higher temperature and longer exposure of heat was required in the lamb to create the same level of injury seen in the fetus, due to increased thickness of the postnatal skin and the increased presence of wool follicles. As for the fetuses, the 15 lambs were also serially euthanized at the same points after scalding, in triplicate.

At each time point, tissue was collected from the scalded area and also from a control, unburned area on the same animal. The tissue was fixed in 4% formalin, processed, and embedded in paraffin. For this study, all sections were cut to 7 μm thickness to examine collagen morphology using Picrosirius dye.

Picrosirius staining

Sections were dewaxed in 100% xylene, followed by washing in 100% ethanol and water. The slides were then incubated in 0.1% Sirius Red F3BA (BDH Laboratory Supplies, Poole, UK) in saturated picric acid for 1 hour at room temperature. After washing in water, they were placed in 0.1 N HCl for 2 minutes followed by another wash in water. The sections were then dehydrated through ethanol and xylene before being permanently mounted in EUKITT (Lomb Scientific, Taren Point, NSW, Australia).

Microscopy and image capture

To visualize the birefringent collagen, a Nikon EP600 microscope (Nikon Corporation, Kanagawa, Japan) was fitted with a polarizing filter (Nikon Corporation, Kanagawa, Japan), through which the background was black and the stained collagen fibers displayed as bright green through to red color. To enable an objective and quantitative scoring system, slides were then captured with a Spot RT slider cooled CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) as digital images. Up to five fields were captured from each slide, and all slides were photographed on the

same day to avoid any variability associated with the light source.

Image analysis

Image morphometry was analyzed using ImagePro Plus® image analysis software (Version 4.1.29, Media Cybernetics, Silver Spring, MD), which can automatically calculate the area (square microns) of defined stained proteins (green or red) in each of the sections. Areas of green (type III collagen-like) and red (type I collagen-like) were calculated for each field captured. To define the pixels counted, ranges were selected in the red, green, and blue channels that, through trial and error, selected the color green or red. These same settings were then used for all analyses. The ratios of type III collagen to type I were then calculated for each field (by dividing the area for green by the area for red) and the average and standard error of the mean were calculated for each treatment.

Statistical analysis

A Student's *t*-test was used to determine significant differences between control and burn for each time point, with $P < 0.05$ taken as the level of significance.

RESULTS

Under polarized light, the collagen fibers could be seen clearly in both the fetal and lamb tissue (Figures 1 and 2). As reported by others, thin fibers that birefringed green in the fetal tissue at the early time points (e.g., day 1, 7) were relatively weak.[11] However, as the age of the fetus increased, the fibers became thicker and showed increasing intensity. With increased gestational time, more yellow and red fibers could be seen (possibly indicating the presence of type I collagen). The green fibers (typical of type III collagen) were more often localized to the upper dermis and epidermis, whereas the red and yellow coarse fibers (typical of type I collagen) were localized to the lower, deep dermis, as observed by others.[15] The pattern of distribution of type I collagen was similar to some immunohistochemical staining conducted by our group using a type I collagen antibody (Research Diagnostics Inc, Flanders, NJ; data not shown). Similar trials using a type III collagen antibody from the same manufacturer were unsuccessful, necessitating the use of the Picrosirius-polarization method.

The arrangement of the collagen visible in the sections showed that after burn, the fetal tissue maintained normal tissue architecture, with regularly arranged collagen fibers. However, the lamb tissue after burn showed irregular collagen organization, with poorly defined structure. Fourteen days after the burn injury, the lamb tissue showed considerable hypertrophy, whereas the fetal tissue did not show hypertrophy at any time point after burn.

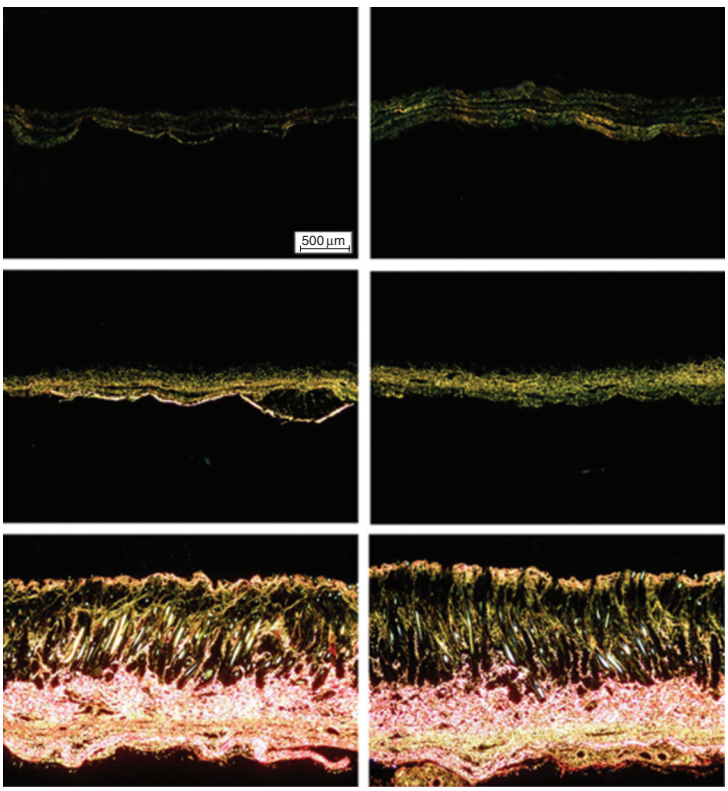

FIGURE 1. Fetal tissue sections stained with Picrosirius dye and viewed under polarized light to detect collagen fibers. All tissue seen in the burn samples is from the scalded area. The control tissue is from an unburned area of skin. (Original magnification × 40)

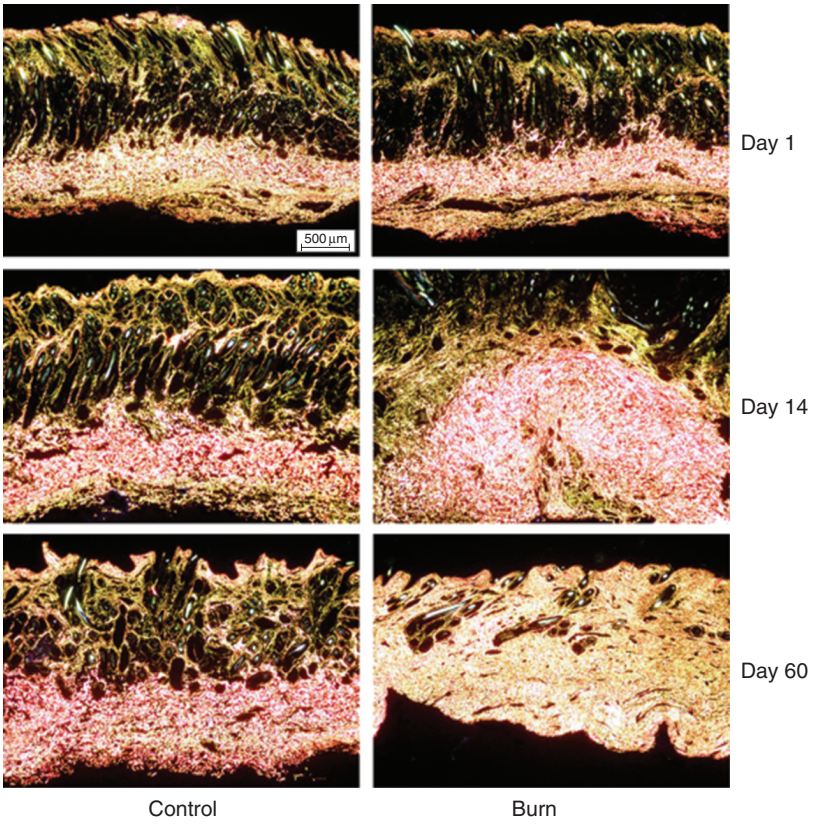

FIGURE 2. Lamb tissue sections stained with Picrosirius dye and viewed under polarized light to detect collagen fibers. All tissue seen in the burn samples is from the scalded area. The control tissue is from an unburned area of skin. (Original magnification × 40)

The fetal tissue over all time points examined contained substantial green staining (Figure 1) and when the ratios of green to red protein were determined, the fetal tissues all had relatively high ratios (Figure 3), with the percentage of total collagen that was green being between 51% and 95% for days 1–21. The day 60 fetal tissue had considerably lower green to red ratios (~0.2) than other fetal time points, similar to those seen in the lamb tissue (0.1–0.3; Figure 4). There were no statistically significant differences between control and burn green to red ratios for any fetal time points, although there appeared to be a trend for more type III collagen to be present in burn tissue.

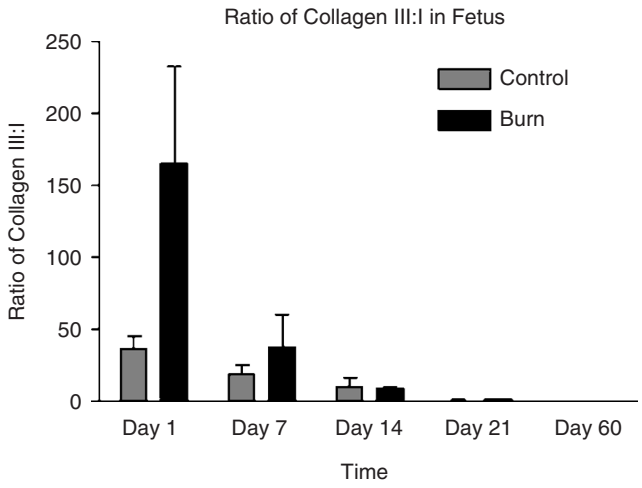

FIGURE 3. Ratios of green (indicative of type III collagen) to red (indicative of type I collagen) staining in the fetal tissue over all time points. Results are represented as mean ± standard error of the mean. Average ratios ranged from 0.2 (day 60 control) to 166 (day 1 burn).

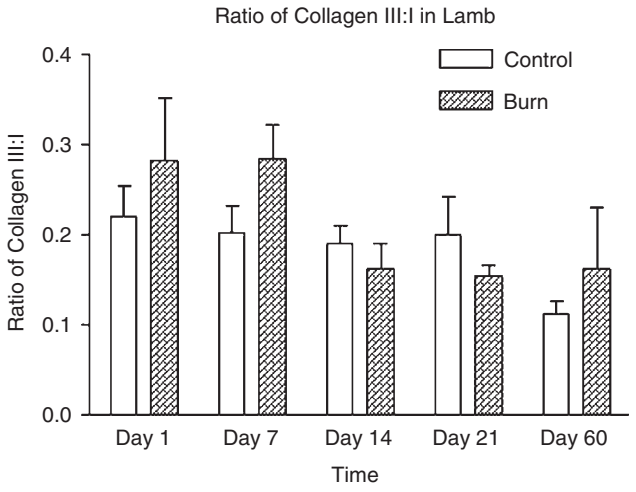

FIGURE 4. Ratios of green (indicative of type III collagen) and red (indicative of type I collagen) staining in the lamb tissue over all time points. Results are represented as mean ± standard error of the mean. Average ratios ranged from 0.1 (day 60 control) to 0.3 (day 7 burn).

The postnatal lamb tissue showed considerably more of the yellow and red thick fibers than the fetal tissue (Figure 2) and had ratios of type III collagen to type I that were much lower than those of the fetus (Figure 4). There were no statistically significant differences between control and burn green to red ratios for any lamb time points.

The total amount of collagen in the fetal tissue increased with age. At day 1 (day 81 gestation), the total collagen was approximately 0.4% that of the newborn lamb (30-days-old) (Figure 5). The collagen levels increased throughout all ages of the fetus, whereas the amount of collagen in the lamb did not alter greatly between different time points.

Finally, a profile of the collagen deposition over time was obtained by calculating the difference in total collagen between each time point. In the fetus (Figure 6), more collagen was deposited with increasing age. There also appeared to be a trend for the burn tissue to be depositing more collagen than control, unwounded tissue. In the lamb (Figure 7), the amount of collagen deposition varies between time points, which is likely to be due to collagen being either deposited or degraded. The hypertrophy of lamb tissue seen macroscopically 14 days after burn can be seen here as a large increase in collagen deposition between days 7 and 14, however, this is not statistically significant.

DISCUSSION

The results from the use of Picrosirius dye in wound healing studies should be viewed with care as regrowing fibers will be small and thin and will therefore display a greenish color, perhaps being misrepresented as type III collagen.[12,16] Likewise, in young, developing tissues the collagen fibers will also be small, again leading to the overrepresentation of type III collagen. In the case here, where there is fetal and neonatal sheep, burned and undamaged tissue, the observer must be careful of what conclusions are drawn. For this reason, proteins that show up as green under polarized light cannot be identified as uniquely type III collagen, but only as "type III collagen-like." For the purposes of this discussion, all future references to type I collagen or type III should be interpreted as "type I or III collagen-like."

This study shows that there is a gestational change in the content and distribution of type III collagen and type I-like fibers. As the fetus matures, more total collagen is produced and over time these collagen fibers begin to thicken and appear red, probably indicating the presence of type I collagen.[16] The majority of collagen present in the day 80 fetus birefringed with a green color, and although we cannot confirm that it is type III collagen that is being detected, we can say that the collagen fibers are smaller and thinner. Whatever the case, it may be that it is these properties of the fibers that assist the fetus to heal without scar.

The profile of collagen content shows that as the fetus increases in age, the collagen deposition also increases. Although there is a trend for there being more type III collagen deposited in the wounded tissue, this is not statistically significant and does not occur at all fetal time points. Likewise, the ratio of type III to I collagen is high in fetal tissue, but there were no statistically significant differences between the ratios for burned and control skin in the fetus. This indicates that the type and amount of collagen are not altered when the fetal wound is healing, but rather that the observed increase in collagen is associated with gestational changes only. This suggests that the extra collagen present in the healing of the fetal wound is representative of the normal growth pattern, rather than a response to an injury.

In the lamb (postnatal animal), there also appears to be a trend for the collagen deposited after wounding to be more like type III collagen, although this was not statistically significant. It is widely accepted that fetal

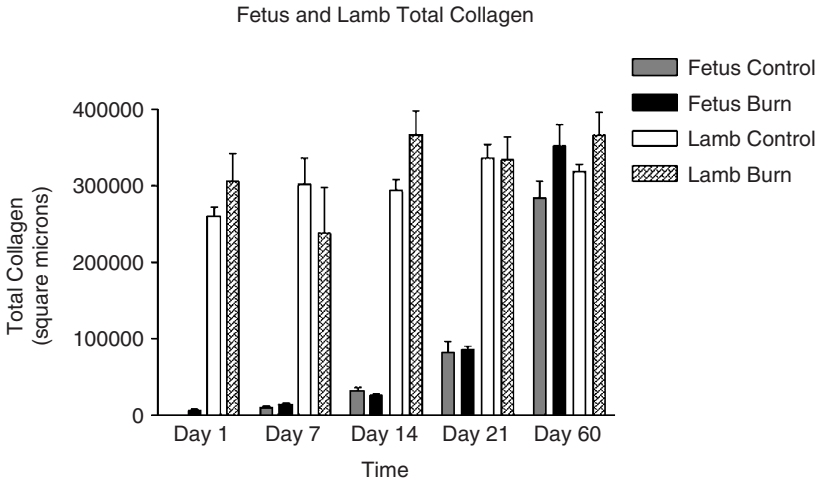

FIGURE 5. Total amount of collagen measured in fetal and lamb tissue, obtained by adding the amount of type I collagen and type III together. Results are represented as mean ± standard error of the mean.

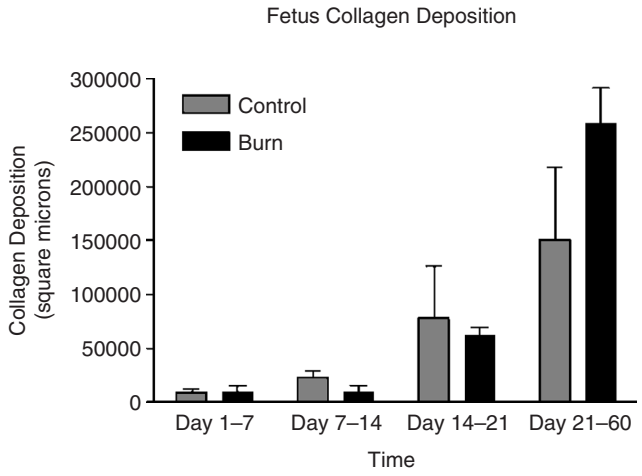

FIGURE 6. Amount of collagen (types I and III) deposited in the fetus between each time point, obtained from the total collagen values. Results are represented as mean ± standard error of the mean.

and adult fibroblasts react differently to transforming growth factor-β–mediated collagen production,[17,18] however, there are conflicting reports in the literature comparing the deposition of different types of collagen in fetal and adult wounds. Gabbiani et al.[19] found that granulation tissue in rat wounds contained proportionally more type IIII collagen. Likewise, Merkel et al.[6] found that adult wounds contained more type III collagen than control skin, and that this increase of type III collagen deposition was higher than in fetal wounds. However, in vitro studies have shown that fetal fibroblasts synthesize more type III and type V collagen than adult fibroblasts.[20] There is no doubt that the model and type of wound play an important role in determining the amount and type of collagen that is deposited. Different models in the literature use incisional wounds, excisional wounds, chemical irritation, and sponge implantation. In the small deep dermal burn wound created in this study, there was little tissue loss, and therefore there was a reduced requirement for collagen deposition. It is likely that a full-thickness burn with a greater loss of tissue would result in a different collagen deposition profile.

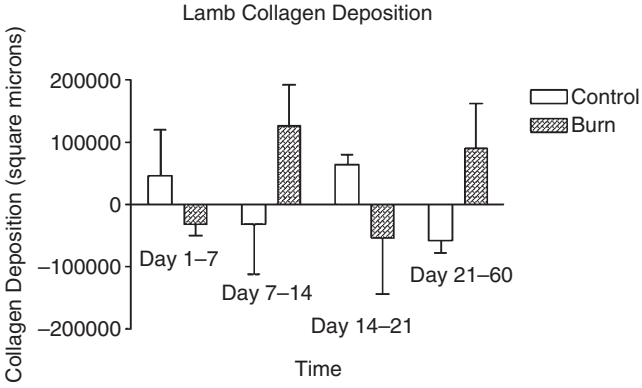

FIGURE 7. Amount of collagen (types I and III) deposited in the lamb between each time point, obtained from the total collagen values. Results are represented as mean ± standard error of the mean.

This study has shown that the amount or type of collagen does not alter in scar formation; however, scarring may be more related to the arrangement of collagen fibers. Examination of Figures 1 and 2 showed that the burnt fetal tissue looked remarkably similar in structure to the control tissue. However, the burned lamb tissue was morphologically different to the control tissue, with collagen that was disordered and without pattern or structure. This became especially obvious in the lamb 14 days after burn. This finding supports those of previous investigators who observed a similar pattern of collagen distribution.[21] In nonscarring fetal sheep incisional wounds, collagen was deposited in a well-organized pattern, parallel to the skin surface. However, in scarring wounds, fibers that were initially laid parallel to the skin surface were packed closely at the wound site in scar bundles at 14 days. The appearance of an altered collagen structure by 14 days is noteworthy as it is at this time point in a clinical setting that an experienced burn surgeon can determine by macroscopic examination if the wound will heal with scar.[22] In the lamb skin 14 days after burn, the hypertrophy seen macroscopically relates to a large increase in collagen deposition; however, at other time points, the amount of collagen deposition varies markedly. The collagen degradation seen may be due to collagen resorption or collagenase digestion during this time. Such activity would help to alleviate the hypertrophy of the lamb tissue 14 days after burn and help maintain the skin structure at other time points. This reorganization and remodeling of collagen during wound healing is suggestive of an autoregulatory process.

The cross-linking pattern of collagen may also be important in the determination of a scar-free wound. Lovvorn et al.[9] found that as age increased, so did the amount of type I collagen cross-linking. Cross-linked peptides became more prevalent in fetal sheep skin after 125 days of gestation, an age associated with the transition from scarless wound healing to healing with scar. In our study, the amount of cross-linking was not measured, but the temporal relationship between gestational age and the transition to a scarring phenotype is similar. The fetal tissue at day 60 (day 140 of gestation) is very similar to the lamb tissue, both in appearance and with similar collagen ratios, whereas the fetal tissue at day 21 (day 101 of gestation) is similar to the younger fetal time points. Therefore, in this model the transition to the scarring phenotype happens between day 101 and 140 of gestation (term = 145 days).

In this study, the Picrosirius polarization method was used to examine the collagen amount and

structure in healing burn wounds. Wound healing is a dynamic process, which continues long after the initial injury has occurred. To fully understand this process, the actual healing wound (and not an artificial environment) must be examined over a period of time, such as the time course used in this study. To only study the wound 1 day, or 14 days after the stimulus, is to study an incomplete picture. Here, the results show that a scarless fetal wound does not heal by significantly altering the type or amount of collagen deposition, but rather, that the healing seen is more representative of the normal fetal growth pattern.

ACKNOWLEDGMENTS

The authors thank the Royal Children's Hospital Foundation (Brisbane, Australia) for its financial support.

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